RESEARCH COMMUNICATION

De novo DNA methylation by Dnmt3a and Dnmt3b is dispensable for nuclear reprogramming of somatic cells to a pluripotent state

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Induced pluripotent stem cells (iPSCs) are generated from somatic cells by the transduction of defined transcription factors, and this process involves dynamic changes in DNA methylation. While the reprogramming of somatic cells is accompanied by demethylation of pluripotency genes, the functional importance of de novo DNA methylation has not been clarified. Here, using loss-of-function studies, we generated iPSCs from fibroblasts that were deficient in de novo DNA methylation mediated by Dnmt3a and Dnmt3b. These iPSCs reactivated pluripotency genes, underwent self-renewal, and showed restricted developmental potential that was rescued upon reintroduction of Dnmt3a and Dnmt3b. We conclude that de novo DNA methylation by Dnmt3a and Dnmt3b is dispensable for nuclear reprogramming of somatic cells.

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Pluripotent stem cells can be generated from somatic cells by viral transduction of the four transcription factors Oct4, Sox2, Klf4, and c-Myc. These induced pluripotent stem cells (iPSCs) are similar to embryonic stem (ES) cells expressing pluripotency genes such as *Nanog* and *Oct4*, and are capable of differentiation into cells of all three germ layers in teratoma assays. Furthermore, mouse iPSCs contribute to the germline in chimeric mice and “all iPSC” mice can be derived through tetraploid complementation (Takahashi and Yamanaka 2006; Maherali et al. 2007; Okita et al. 2007; Wernig et al. 2007; Boland et al. 2009; Kang et al. 2009; Zhao et al. 2009). iPSCs generated from human sources are thought to be useful for drug discovery and regenerative medicine (Yamanaka 2009). In fact, in two proof-of-principle studies, iPSCs have been shown to alleviate symptoms in mouse models of sickle cell anemia and Parkinson’s disease (Hanna et al. 2007; Wernig et al. 2008).

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The molecular events leading from a differentiated to a pluripotent cell during iPSC derivation are only emerging and remain largely unknown (Hochedlinger and Plath 2009). Studies have shown that distinct markers are activated or repressed sequentially after the induction of reprogramming, with the repression of differentiation genes occurring early and the activation of genes essential for pluripotency, such as *Oct4* or *Nanog*, occurring late during this process (Brambrink et al. 2008; Stadtfeld et al. 2008). The epigenetic state of the differentiated donor cell is completely reprogrammed, and DNA methylation and histone marks of the iPSC resemble that of ES cells (Maherali et al. 2007; Wernig et al. 2007; Mikkelsen et al. 2008).

Changes in DNA methylation patterns are essential for successful nuclear reprogramming, exemplified by the necessity for loss of promoter methylation in pluripotency genes in iPSCs (Takahashi and Yamanaka 2006). If loss of DNA methylation is not achieved, the cells will be only partially reprogrammed (Mikkelsen et al. 2008). Several studies have investigated de novo DNA methylation during the reprogramming process. For example, it has been documented that de novo DNA methyltransferases are highly induced upon reprogramming, and it is known that mouse ES cells have a very high expression of these epigenetic regulators (Okano et al. 1998; Stadtfeld et al. 2008). Also, two studies found increased DNA methylation in certain genomic regions in iPSCs, with the functional importance of this finding remaining elusive (Deng et al. 2009; Doi et al. 2009). However, it has not been resolved whether de novo DNA methylation is important or might even be essential for nuclear reprogramming of somatic cells to a pluripotent state.

De novo DNA methylation in mammals is mediated by Dnmt3a and Dnmt3b (Li 2002) and knockout studies in mice have shown that de novo DNA methylation is essential for development, since *Dnmt3a*-deficient animals die several weeks after birth and *Dnmt3b*-deficient animals die in utero (Okano et al. 1999). Further experimentation has shown that fibroblasts lacking *Dnmt3b* are prone to spontaneous immortalization and genomic instability (Dodge et al. 2005), although mouse ES cells can tolerate the complete absence of DNA methylation (Meissner et al. 2005). DNA methylation by Dnmt3a has a crucial role for imprinting in development (Kaneda et al. 2004).

In this study, we sought to investigate the role of Dnmt3a and Dnmt3b in the generation of iPSCs. Using mouse embryonic fibroblasts (MEFs) in which *Dnmt3a* and *Dnmt3b* were conditionally deleted through Cre recombinase, we show that nuclear reprogramming is achieved in the absence of the de novo DNA methyltransferases Dnmt3a and Dnmt3b.

Results and Discussion

Generation of iPSCs in the absence of the de novo DNA methyltransferases Dnmt3a and Dnmt3b

To ask whether somatic cells can be reprogrammed into a pluripotent cell in the absence of de novo DNA methylation, we used MEFs that were homozygous for floxed alleles of both *Dnmt3a* and *Dnmt3b*. Two

complementary approaches were used to generate iPSCs (Fig. 1A). In the first approach, we transduced double-conditional MEFs (referred to as 3ab MEFs) with lentiviruses encoding the reprogramming factors Oct4, Sox2, c-Myc, and Klf4 and the reverse tetracycline transactivator M2rtTA (Brambrink et al. 2008; Hockemeyer et al. 2008). After transduction, the cells were infected with an adenovirus containing a Cre recombinase and GFP. FACS (fluorescence-activated cell sorting) was used to isolate GFP-positive cells, and reprogramming was initiated 1 d later by adding doxycycline (DOX) to the medium. Since it is known that *Dnmt3a* and *Dnmt3b* are expressed at low levels in somatic cells (Okano et al. 1998), we aimed to exclude the possibility of remaining functional *Dnmt3a* and *Dnmt3b* after the initiation of reprogramming. To this end, in the second approach, 3ab MEFs were first infected with the adenovirus harboring the Cre recombinase, passaged several times, and then transduced with the individual reprogramming factors. Similarly to wild-type MEFs, addition of DOX to 3ab MEFs induced morphological changes within several days in both approaches (Supplemental Fig. S1A). After 17–28 d of DOX treatment, single colonies morphologically resembling ES cells were subcloned. To select for DOX-independent clones, DOX was withdrawn from the medium shortly after subcloning.

To characterize 3ab-deficient iPSCs, immunostaining was performed. 3ab-deficient iPSCs were found to be positive for the stem cell markers alkaline phosphatase, SSEA-1, Oct4, and Nanog (Fig. 1B; Supplemental Fig. S1B–D), and the staining patterns were indistinguishable from those of control V6.5 ES cells. The expression of the pluripotency gene *Nanog* was also investigated by quan-

titative RT-PCR (qRT-PCR) and was found to be expressed in 3ab-deficient iPSCs at levels comparable with ES cells (Supplemental Fig. S1E). These results suggest that reprogramming can occur in the absence of de novo DNA methylation through *Dnmt3a* and *Dnmt3b*.

Upon differentiation of ES cells, pluripotency genes such as *Oct4* and *Nanog* are highly methylated, reflecting stable long-term silencing (Feldman et al. 2006), while iPSC generation leads to demethylation, consistent with reactivation of *Oct4* and *Nanog*. As expected, bisulfite sequencing demonstrated that the *Nanog* gene became demethylated in 3ab-deficient iPSCs, comparable with V6.5 ES cells (Supplemental Fig. S1F).

To evaluate whether the *Dnmt3a* and *Dnmt3b* alleles had been deleted by Cre recombinase, Southern blot analyses were performed. Individual colonies were subcloned from iPSCs derived from FACS-isolated Adeno-Cre-GFP-infected MEFs. Figure 2A shows that all *Dnmt3a* and *Dnmt3b* alleles were deleted, confirming that iPSCs can be derived in the absence of de novo DNA methylation. Similarly, >20 subclones generated with the second approach had deleted all *Dnmt3a* and *Dnmt3b* alleles (Fig. 2B).

To confirm the independent origin of 3ab-deficient iPSC lines, we performed Southern blot analysis with a probe that recognizes both endogenous and transgenic *Oct4*. The majority of the 18 iPSC lines randomly chosen for this analysis had a unique integration pattern and thus were of independent origin (Supplemental Fig. S2A). The number of integrations for the *Oct4* provirus varied between two and eight, with clones #8 and B2 having the lowest and clone A6 having the highest number of integrations. Expression analysis of *Oct4* proviral transcription correlated with basal vector expression, with clone A6 showing some residual transgene expression (Supplemental Fig. S2B). Also, we observed different numbers of proviral integrations for the other reprogramming factors, with clone B2 lacking a c-Myc proviral integration (Supplemental Fig. S3).

It is known from nuclear transfer studies that hypomethylation of somatic donor cells increases reprogramming efficiency (Blelloch et al. 2006). To ask whether reprogramming was enhanced in the absence of de novo DNA methylation, we infected 3ab conditional MEFs with either an Adeno-GFP or an Adeno-Cre-GFP virus and sorted equal amounts of GFP-positive cells. Then, we induced reprogramming by transduction with a polycistronic vector harboring the four reprogramming factors (Sommer et al. 2009). After 16 d of reprogramming, we omitted DOX and counted colonies on day 19. We observed only a slight difference in colony number between control and 3ab-deficient samples (1.5-fold), suggesting that there is no major effect on reprogramming in the absence of de novo DNA methylation. One representative plate was stained for alkaline phosphatase, a commonly used pluripotency marker (Supplemental Fig. S4).

Molecular characterization of 3ab-deficient iPSCs

It was shown previously that a global change in DNA methylation patterns occurs during iPSC derivation (Mikkelsen et al. 2008). We therefore investigated the methylation of genomic repeat sequences during reprogramming in the absence of de novo DNA methylation. To this end, we compared 3ab-deficient with control iPSCs or V6.5 ES cells. Southern blot analysis with the

Figure 1. Generation of iPSCs in the absence of de novo DNA methyltransferase activity. (A) Schematic of the two approaches to reprogram conditional knockout MEFs for *Dnmt3a* and *Dnmt3b*. MEFs were infected with adenovirus harboring a Cre recombinase and GFP either after or before infection with inducible lentiviruses encoding the reprogramming factors Oct4, Sox2, c-Myc and Klf4. (B) Immunostaining for the pluripotency marker Nanog. Bars, 100 μ m.

Figure 2. Characterization of 3ab-deficient iPSC lines. (A) Southern blot analysis shows 3ab-deficient iPSC lines obtained through approach 1 (Fig. 1A). Clone #8 is indicated by an asterisk. (B) Southern blot analysis of 3ab-deficient iPSC lines obtained through approach 2 (Fig. 1A). Clones A6 and B2 are indicated by an asterisk. Additionally, 3ab MEFs infected with Adeno-Cre-GFP are shown before the transduction with the reprogramming factors and over the course of several passages (1–3). Lane 4 displays DNA from a culture dish containing mixed iPSC colonies. Note that the loop-out induced by Cre recombinase was highly efficient. “2lox” represents the conditional alleles and “1lox” represents the deficient alleles of *Dnmt3a* and *Dnmt3b*, respectively.

methylation-sensitive enzyme HpaII showed that 3ab-deficient iPSC lines had slightly decreased DNA methylation levels of minor satellite repeats and intracisternal A particles (IAPs) (Supplemental Fig. S5), consistent with previous results showing reduced IAP methylation in *Dnmt3a* and *Dnmt3b* mutant cells (Okano et al. 1999). Since it has been demonstrated that female ES cells are hypomethylated (Zvetkova et al. 2005), we determined the gender of the 3ab-deficient iPSC lines and found that male clone #8 and female clones A6 and B2 had similar methylation levels of the genomic repeat sequences.

To investigate global gene expression in 3ab-deficient iPSCs, we performed expression profiling (Supplemental

Fig. S6). This analysis revealed that 165 genes were >2.5-fold up-regulated in 3ab-deficient iPSCs and 116 genes were 2.5-fold down-regulated in comparison with control iPSC lines (Supplemental Table S1). A large proportion of the up-regulated genes were X-linked, such as genes of the *rhox* cluster, a known target for *Dnmt3a* and *Dnmt3b*. In fact, loss of *Dnmt3a* and *Dnmt3b* in ES cells leads to activation of the *rhox* cluster, which is normally silenced (Oda et al. 2006). Furthermore, it was shown that ES cells that lack both de novo and maintenance DNA methyltransferases up-regulate genes on the X chromosome (Fouse et al. 2008). We found *Gtl2* and *Rian*, two members of the *Dlk1-Dio3* cluster, up-regulated in 3ab-deficient iPSCs, indicating that *Dnmt3a/b*-mediated DNA methylation may be involved in the regulation of this genomic region, as suggested previously (Kato et al. 2007). For functional annotation, we performed gene ontology (GO) analyses of the differentially regulated genes in 3ab-deficient iPSCs (Supplemental Table S2). Top GO terms for the up-regulated genes were chromosome organization (GO: 0051276) and organelle organization (GO: 0006996). However, more specialized categories such as meiosis (GO: 0007126) or reproductive cellular process (GO: 0048610) were also observed, and it is known that *Dnmt3a* plays an important role in germ cell development (Kaneda et al. 2004; Kato et al. 2007). The top GO terms for the down-regulated genes were in response to stimulus (GO: 0050896) and multicellular organismal development (GO: 0007275), with other more specialized developmental GO terms. It was noteworthy that up-regulated genes would rather be grouped into the intracellular region (GO: 0005622), whereas down-regulated genes would fall into the extracellular region (GO: 0005576). In summary, our data suggest that 3ab-deficient iPSCs display an expression profile highly similar to control iPSCs, yet with some noticeable differences.

A previous study grouped gene promoters in mouse ES cells into either methylated or unmethylated (Fouse et al. 2008). We compared our gene list of differentially expressed genes in 3ab-deficient iPSCs with the gene list of methylated and unmethylated promoters to evaluate whether we could identify a correlation between the lack of de novo DNA methylation and the observed expression profile. We found that 59 of the differentially expressed genes in 3ab-deficient iPSCs are methylated and 55 genes are unmethylated in mouse ES cells (Supplemental Table S3). Using a different data set (Meissner et al. 2008), we did not observe a preferred distribution of the differentially regulated genes into methylated or unmethylated in normal mouse ES cells in comparison with MEFs

(Supplemental Fig. S7). Additionally, sites of enhanced DNA methylation in human iPSCs in relation to human ES cells were reported previously (Doi et al. 2009). However, differentially expressed genes in 3ab-deficient iPSCs did not significantly overlap with these sites. These data suggest that, while expression of some genes in ES cells may be affected by the state of DNA methylation, this effect may not be direct.

Developmental potential of *Dnmt3a*- and *Dnmt3b*-deficient iPSCs

To assess developmental potential, 3ab-deficient iPSCs were injected into the flanks of SCID (severe combined immunodeficient) mice. The resulting teratomas showed mostly ectodermal structures and few endodermal-like, but no well-differentiated, mesodermal structures (Fig. 3A), suggesting that 3ab-deficient iPSCs have only a restricted developmental potential. Furthermore, the overall size of teratomas derived from 3ab-deficient iPSCs was considerably smaller than that of teratomas from control V6.5 ES cells (data not shown) or control 3ab⁺ iPSCs that were able to contribute to all three germ layers (Supplemental Fig. S8). This is in agreement with previous studies demonstrating that high-passage *Dnmt3a*- and *Dnmt3b*-deficient ES cells were unable to form teratomas, likely because of a decreased level of global DNA methylation. Consistent with this conclusion, developmental potential in *Dnmt3a/3b*-deficient ES cells could be restored by reintroduction of *Dnmt3a* and *Dnmt3b* and reestablishment of DNA methylation patterns (Chen et al. 2003).

To investigate whether developmental potential could be restored to 3ab-deficient iPSCs, we reintroduced functional *Dnmt3a* and *Dnmt3b* by lentivirus-mediated gene transfer (Supplemental Fig. S9). Indeed, the rescued lines formed teratomas that displayed differentiated cells from all three germ layers (Fig. 3A). This observation suggested that 3ab-deficient iPSCs had been reprogrammed yet could not differentiate, merely due to the

lack of *Dnmt3a* and *Dnmt3b* that were rescued upon their reintroduction. As a more stringent assay for pluripotency, we injected eGFP-marked 3ab-deficient iPSCs and rescued lines into blastocysts and harvested the resulting embryos at mid-gestation (Supplemental Fig. S9). We were able to obtain chimeric embryos with clearly visible eGFP signals, indicative of a contribution from the rescued lines, underscoring their developmental potential (Fig. 3B). The degree of chimerism varied between individual embryos. 3ab-deficient iPSCs did not contribute to developing embryos based on the lack of an eGFP signal (data not shown), which was confirmed by the absence of immunohistochemical staining for GFP (Supplemental Fig. S10). In some high-contribution chimeras from the rescued lines, developmental malformations were observed (Fig. 3B) that are likely due to constitutive expression of the lentivirus-transduced *Dnmt3a* and *Dnmt3b* genes. In summary, reintroduction of *Dnmt3a* and *Dnmt3b* restored pluripotency to the *Dnmt3a*- and *Dnmt3b*-deficient iPSCs.

Conclusions

In this study, we used conditional knockout MEFs to generate iPSCs in the absence of de novo DNA methylation through *Dnmt3a* and *Dnmt3b*. We observed robust reactivation of pluripotency markers in 3ab-deficient iPSCs, and their derivation appeared to be similarly efficient compared with wild-type cells. The methylation status of genomic repeat sequences in 3ab-deficient iPSCs was slightly decreased. The global expression profile of 3ab-deficient iPSCs closely resembled that of control iPSCs, although the expression of a small number of genes was changed in 3ab-deficient iPSCs. The question of how gene silencing is achieved in 3ab-deficient iPSCs remains a particularly interesting one. A recent study suggested that gene silencing in mouse ES cells can be achieved by alternative mechanisms to DNA methylation, such as histone methylation by ESET (also known as KMT1E) (Matsui et al. 2010). Whether such mechanisms

assure gene silencing in 3ab-deficient iPSCs deserves further study. 3ab-deficient iPSCs formed teratomas that were smaller than teratomas from control lines and had a restricted developmental potential. However, developmental potential could be rescued upon reintroduction of *Dnmt3a* and *Dnmt3b* into 3ab-deficient iPSCs, consistent with de novo DNA methylation mediated by *Dnmt3a* and *Dnmt3b* being crucial for normal development. In summary, our results provide evidence that the genome of a differentiated cell that is deficient for this major epigenetic regulatory system can be reprogrammed to a pluripotent state.

Figure 3. 3ab-deficient iPSCs form teratomas with a restricted developmental potential. (A) Teratomas from #8 and B2 3ab-deficient iPSCs. Reintroduction of functional *Dnmt3a* and *Dnmt3b* into 3ab-deficient iPSCs rescues the ability to efficiently form cells from the three germ layers in a teratoma assay. (B) eGFP-marked rescue lines were injected into blastocysts, and resulting chimeric embryos at mid-gestation are shown. The eGFP signal was clearly visible in the majority of embryos, whereas the degree of chimerism varied. Two embryos with developmental defects are shown. The first embryo of the bottom panel did not show any eGFP signal and serves as a reference of background.

Materials and methods

Cell culture and iPSC derivation

MEFs were isolated from double-homozygous mice for the conditional alleles of *Dnmt3a* and *Dnmt3b* (Lin et al. 2006; Nguyen et al. 2007). iPSCs were generated with inducible lentiviruses

as reported previously (Brambrink et al. 2008; Hockemeyer et al. 2008). Recombination of the conditional alleles of *Dnmt3a* and *Dnmt3b* was induced by infection with adenovirus harboring Cre recombinase and GFP (Ad5CMVCre-eGFP, Gene Transfer Vector Core, University of Iowa). Control iPSC lines were derived from 3ab MEFs without infection with Adeno-Cre-GFP. However, for the efficiency experiment using a previously described DOX-inducible polycistronic vector (Sommer et al. 2009), we included an adenovirus containing only GFP (Ad5CMV-eGFP) to generate the control sample.

Generation of rescue lines and targeting of the *Rosa26* locus.

Full-length *Dnmt3a* and *Dnmt3b* were introduced with lentiviral vectors into 3ab-deficient iPSC lines. Both 3ab-deficient and rescue lines were targeted in the *Rosa26* locus with eGFP for the purpose of blastocyst injections.

Western blot analysis

The antibodies to detect *Dnmt3a* and *Dnmt3b* were purchased from Imgenex (IMG-268A and IMG-184A).

Southern blot analysis

Southern blot analyses were used to investigate the loop-out status of the *Dnmt3a* and *Dnmt3b* conditional alleles using previously described probes (Lin et al. 2006; Nguyen et al. 2007). As probes for proviral integration analyses, fragments of the cDNAs for the reprogramming factors were excised from the respective lentiviral vectors. The probes for minor satellite repeats (pMR150) and IAPs were described previously (Chapman et al. 1984; Walsh et al. 1998).

Immunostaining and immunohistochemistry

Immunostaining was described previously (Wernig et al. 2007). Antibodies used were for Oct4 (C10, Santa Cruz Biotechnology), SSEA-1 (Developmental Studies Hybridoma Bank), and Nanog (A300-397A, Bethyl Laboratories). Alkaline phosphatase staining was performed with the Vector Red substrate kit (Vector Laboratories) according to the manufacturer's instructions. The antibody against GFP was ab290 (Abcam). Images for the immunostainings were taken with a Nikon Eclipse Ti microscope and processed equally between samples with Photoshop.

Bisulfite sequencing

Two micrograms of phenol-chloroform-extracted genomic DNA was converted with bisulfite using the Epitect kit from Qiagen. The gene-specific PCR primers recognizing bisulfite-treated DNA were described previously for *Nanog* (Imamura et al. 2006). PCR-amplified products were gel-purified and cloned into TOPO pCR2.1 from Invitrogen and sequenced with M13 primers.

qRT-PCR analysis

Primers for *Nanog* qRT-PCR analysis were described previously (Stadtfeld et al. 2008). Primers used to detect total *Oct4* were described elsewhere (Hanna et al. 2009). Primers to detect transgenic *Oct4* were GCTCAGT GATGCTGTGATCAGG (forward) and CAAAGGCATTAAGCAGCG TATC (reverse).

Expression profiling

Total RNA was isolated with the RNeasy kit from Qiagen. RNA was labeled with the QuickAmp kit from Agilent and hybridized to 4x44k mouse whole-genome arrays from Agilent. The two-color microarrays were normalized across channels by lowess (locally weighted scatter plot smoothing) and between arrays by quantile normalization of average intensities (Aquantile), as implemented by the limma package in Bioconductor. Differential expression was defined by an expression ratio of at least 2.5-fold. To reduce any potential gender bias, expression ratios were calculated giving equal weight to male (#8) and female (mean of A6 and B2)

3ab-deficient iPSC lines and to male (mean of #3, #4, and #5) and female (#6) control iPSC lines. The scatter plot (Supplemental Fig. S6) highlights probes that are at least 2.5-fold differentially expressed in 3ab-deficient iPSC lines compared with controls, as listed in Supplemental Table S1. Microarray data have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE28629.

GO analyses

Functional annotation was performed with the Database for Annotation, Visualization, and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov>). As a cutoff for the functional categories, we chose an FDR (false discovery rate) of 10.

Teratoma formation assays

Teratomas were generated by injecting iPSCs and control cells into the flanks of SCID mice. Teratomas were harvested, fixed with 10% formalin overnight, and sectioned for H&E staining.

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